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Three-Dimensional Ovarian Cancer Spheroids

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14. ABSTRACT Background: Ovarian cancer metastasis involves growth of ovarian cancer cells shed from primary tumor as multicellular spheroidal aggregates within ascites fluid. Multicellular tumor spheroids are physiologically significant 3D <i>in vitro</i> models for preclinical drug screens. Conventional hanging drop spheroid cultures utilize high number of starting cells, and are tedious for long-term maintenance. Objective: The objective of our study was two fold: A) to generate stable, uniform multicellular spheroids using very small number of ovarian cancer cells in a novel 384-well hanging drop platform and to B) establish a high throughput 3D platform for preclinical studies. Methods: We used non-serous and serous ovarian cancer cells as well as patient ascites to demonstrate stable incorporation of as few as 10 cells into a single spheroid. Results: Spheroids had uniform geometry, and their projected spheroid areas varied as a function of initial cell seeding density. Cell-cell interaction was detected using cytoskeletal actin staining. Nuclei counterstaining indicated compaction of cells forming tightly packed spheroids, with demarcated boundaries. Cells within spheroids demonstrated high viability of >85%. Spheroids remained 80% viable in response to 50µM cisplatin, whereas 2D monolayer cultures were only 30% viable, suggesting multicellular ovarian cancer spheroids are inherently chemoresistant. Conclusions: Ovarian cancer spheroids can be generated from limited cell numbers in high throughput hanging drops with high viability. 3D spheroids demonstrate therapeutic resistance relative to cells in 2D culture. Stable incorporation of low cell numbers is advantageous when translating this research to rare populations of cancer stem-like cells isolated from patient samples. Our platform is applicable to understanding ovarian cancer biology and for carrying out preclinical drug sensitivity assays.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	10
3. Overall Project Summary.....	11
4. Key Research Accomplishments.....	15
5. Conclusion.....	33
6. Publications, Abstracts, and Presentations.....	34
7. Inventions, Patents and Licenses.....	36
8. Reportable Outcomes.....	37
9. Other Achievements.....	39
10. References.....	44
11. Appendices.....	49

Introduction

Chemosensitivity assays are currently available for ovarian cancer. Although potencies of anticancer drugs when tested in conventional two-dimensional (2D) plastic cultures *in vitro* are favorable¹⁻⁴, resistance is often observed in patients. This is likely in part because **standard culture conditions on rigid, flat and adherent 2D surfaces do not recapitulate the *in vivo* microenvironment**; as they lack structural cues, induce genetic changes and non-physiological cellular signaling in tumor cells. This often results in less accurate predictions of drug efficacy⁵⁻⁸. Furthermore, standard chemotherapy assays typically assess response of the bulk population of tumor cells. However, it is becoming increasingly clear that only a minority of cancer cells with stem cell-like properties are responsible for maintenance and growth of epithelial ovarian tumors. These cells, known as cancer stem cells (CSCs) or cancer initiating cells (CICs), are hypothesized to be responsible for recurrence of the disease after standard clinical care, mediated by their inherent chemoresistance associated with cellular quiescence and expression of drug membrane transporters⁸⁻¹¹. **This suggests that current *in vitro* models of ovarian cancer used for preclinical testing are inadequate. The inability of standard assays to predict therapeutic responses of CSCs suggest an unmet need for development of *in vitro* models that can more accurately recapitulate the tumor microenvironment and assess the responses of rare CSCs.** Interestingly, one of the phenotypes of chemotherapy resistant ovarian CSCs (OvCSCs) is the ability to grow in suspension as tumor spheroids^{12, 13}. Growth in suspension mirrors the *in vivo* growth of ovarian cancer cells in ascites in patients¹⁴. **We hypothesize that CSC derived tumor spheres grown in suspension will more accurately mimic *in vivo* ovarian cancer growth and will therefore be a more accurate model to screen**

for effective anti-cancer therapeutics. We therefore propose to develop 3D OvCSC spheroids model for drug testing (see Figure 1).

Platforms for 3D cell culture, such as scaffolds, hydrogels, and microfluidics can provide enhanced models for testing of drug toxicity, delivery, and metabolism compared to conventional 2D cultures, due to their more physiological cell-cell contact geometry, mass transport, and mechanical properties¹⁵. Among these, spheroids are one of the most common and versatile methods of culturing cell in 3D¹⁶. Consistent with our hypothesis, spheroids are typically more resistant to chemotherapy and radiotherapy compared to cells cultured as 2D monolayers¹⁷. Spheroids present a more physiological platform for studying ovarian cancers due to the following reasons:

1) Spheroids model the 3D architecture of the ovarian tissue, including multicellular arrangement and extracellular matrix deposition, found *in vivo*. Such arrangements, which are absent in conventional culture formats, have **diffusional limits to mass transport of drugs, nutrients and other factors, similar to *in vivo* tissues**. Due to their mimicry of the physiological barriers to drug toxicity and delivery *in vivo*, spheroid can serve as an improved assay format for testing these. 2) Being closely packed 3-D structures; **spheroids have sizeable cell-cell interactions, including tight junctions, comparable to those in *in vivo* ovarian tissues**. These cell-cell contacts and ensuing communication have been found to influence response of cells to drugs^{18, 19}. 3) Co-culture spheroids can be formed with two or more cell types in varying ratios representing intercellular signaling and architecture seen *in vivo*. These **co-culture spheroids can help decipher how multiple cell types found in tissues *in vivo* might impact carcinogenesis, metastasis and drug toxicity**. 4) Rare primary OvCSCs may be incorporated and maintained in spheroids, which can facilitate targeting these specific cells with drugs. It is often difficult to

maintain small numbers of such cells in conventional culture formats, and to decode their independent response to the drug and delivery mechanism. Compared with conventional culture, **spheroid cultures not only enrich stem cell subpopulations, but also maintain key properties of stem cells, including gene expression profiles, colony-forming and/or tumorigenic activity, differentiation potential, cytokine secretion, and resistance to chemotherapy²⁰⁻⁴⁰**. Thus, spheroids represent a physiological model for the rare CSCs, and present a great opportunity for therapeutic intervention targeting these cells.

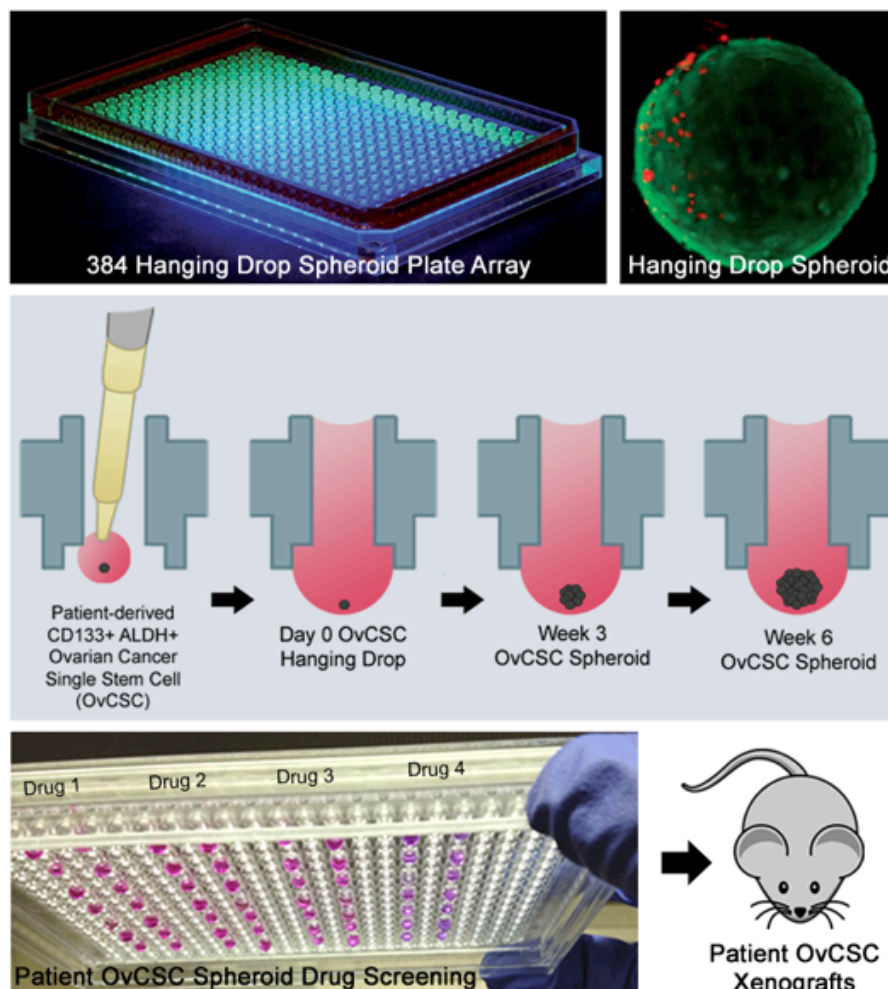


Figure 1: High throughput hanging drop platforms for three dimensional ovarian cancer spheroids models of drug screening and personalized medicine. Human ovarian cancer

spheroids will be created from patient derived ovarian cancer stem cells (FACS sorted CD133+ ALDH+) on the 384 HD plate with as few as 1-10 cells. These ovarian cancer stem cell (OvCSC) spheroids will be maintained for up to 6 weeks in our platform and utilized for drug screening and xenograft studies.

Innovation

While spheroid culture for drug screening demonstrates great promise, current methods to form and maintain them for long term are inadequate. Conventionally spheroids are formed via hanging drops, culture of cells on non-adherent surfaces, spinner flask cultures, and NASA rotary cell culture systems⁴¹⁻⁴⁸. In conventional hanging drop method, it is extremely difficult to exchange cell culture media without compromising the integrity of the droplets, which makes long-term culture almost impossible. More advanced spheroid formation methods developed recently often utilize micro- and nano-technologies such as: microfluidic devices, microwells, surface-modified substrates, porous 3D scaffolds, nano-imprinted scaffolds, hydrogel arrays^{24, 43, 44, 49-58}. Such techniques generally provide great improvements over spheroid size-control, cellular composition, and throughput. However, many of these sophisticated platforms are complicated and thus require specially trained users to fabricate and operate the devices. Or they may allow efficient spheroid formation but not allow for convenient prolonged spheroid culture and drug testing where media exchange or reagent additions are required. In addition, many of these devices suffer from material compatibility issues with many hydrophobic compounds such as anti-cancer therapeutic drugs, which limits the applicability of these high-throughput devices to drug screening and testing. It is also challenging to render microscale-based device surfaces non-adherent to cells for long term. As a result, such devices are often not suitable for 3D long-term culture as cells eventually start attaching to device surfaces and thus becoming 2D cultures.

To overcome these problems, we have recently developed a 384-format hanging drop (HD) array plate as a user-friendly solution to spheroid formation and culture⁵⁹⁻⁶¹. Our design allows efficient formation of uniformly sized spheroids that are viable in culture for more than 30 days. The format also offers ease of use for high throughput drug testing with the

capacity for multiplexing (e.g. liquid handling robots and plate readers). Thus, our system offers significant improvements over existing 3D spheroid culture methods⁵⁹⁻⁶¹. Our system has an innovative design that results in consistent geometry of the HD without spreading out, and leads to more robust and stable culturing conditions not possible on conventional flat HD substrates. The cells slowly aggregate in the bottom center of the hanging droplet, and eventually form into a tight spheroid. The access holes on the top of the plate allow direct manipulation of the droplets, greatly simplifying the initial droplet formation and subsequent media exchange procedures by eliminating the tedious HD culture dish inversion required in the conventional HD method. Using this platform we have successfully formed spheroids (mono-, co- and tri-cultures) with varying initial cell seeding density, ranging from 50 cells to 15,000 cells with different cell lines and *primary cells*⁵⁹⁻⁶¹. We have also improved upon the stability of the spheroids formed on the HD array and removed the susceptibility to small mechanical shocks by optimizing the plate design with micro-rings to stabilize droplets against mechanical perturbations and prevent surface fouling. This system enables long-term cell spheroid culture for more than 22 days within the droplet array^{60, 61}. With enhanced droplet stability, the second-generation 384-HD array plates provide new opportunities for high-throughput preparation and maintenance of microscale 3D ovarian cancer stem cell constructs for drug screening and cell analysis.

Keywords

Spheroids, Multicellular tumor spheroids, 3D culture, Ovarian cancer stem cells, Mesenchymal stem cells, Cancer associated mesenchymal stem cells, Ascites, Serum free medium, High Throughput, Preclinical drug testing, Personalized medicine

Overall Project Summary

My lab is working on developing novel *in vitro* three dimensional spheroid model of ovarian cancer for direct testing of drug therapies for human ovarian cancer (OvCa) in a simple and cost-effective manner. Over the last 20 years, no new ovarian cancer drugs have been approved nor have demonstrated effectiveness for clinical use. Pre-clinical drug screening is largely performed in two-dimensional (2-D) cultures of ovarian cancer cells on a stiff plastic substrate. Such conventional 2D plastic cultures confirm potency of anticancer drugs, but resistance to such drugs is observed in patients. Moreover, it is now believed that a minority of cancer cells with stem cell-like properties are sufficient for maintenance and growth of ovarian tumors, and even lead to tumor recurrence after therapeutic intervention. However, standard assays are unable to predict therapeutic responses of cancer stem cells (CSCs). *In vivo*, chemotherapy resistant ovarian CSCs (OvCSCs) can grow in suspension as tumor spheroids. Therefore, we hypothesize that CSC derived tumor spheres grown in suspension will more accurately mimic *in vivo* ovarian cancer growth, a more accurate model to screen for effective anti-cancer therapeutics.

Spheroids present a more physiological platform for studying OvCa stem cells due to the following features:

1. Spheroids have diffusional limits to mass transport of drugs, nutrients and other factors, similar to *in vivo* tissues.
2. Spheroids have sizeable cell-cell interactions, including tight junctions, comparable to those in *in vivo* ovarian tissues.
3. Co-culture spheroids can help decipher how multiple cell types found in tissues *in vivo* might impact carcinogenesis, metastasis and drug toxicity.

4. Rare primary OvCSCs may be incorporated and maintained in spheroids, which can facilitate targeting these specific cells with drugs.
5. Spheroid not only enrich stem cell subpopulations, but also maintain key properties of stem cells, including gene expression profiles, colony-forming and/or tumorigenic activity, differentiation potential, cytokine secretion, and resistance to chemotherapy.

There are many well-established conventional methods to generate spheroids, which include, hanging drops, culture of cells on non-adherent surfaces, spinner flask cultures, NASA rotary cell culture systems. However, these methods have several disadvantages. Conventional hanging drop method: extremely difficult to exchange cell culture media without compromising the integrity of the droplets, which makes long-term culture almost impossible. More advanced spheroid formation methods include: microfluidic devices, microwells, surface-modified substrates, porous 3D scaffolds, nano-imprinted scaffolds, hydrogel arrays. Many of these techniques provide great improvements over spheroid size-control, cellular composition, and throughput. However, they require specially trained users to fabricate and operate the devices. Some of these methods may allow efficient spheroid formation but not allow for convenient prolonged spheroid culture and drug testing where media exchange or reagent additions are required. But they often suffer from material compatibility issues with many hydrophobic compounds such as anti-cancer therapeutic drugs, which limits the applicability of these high-throughput devices to drug screening and testing. Moreover, it is challenging to render microscale-based device surfaces non-adherent to cells for long term. Therefore, none of these methods are amenable for generation of OvCa stem cells spheroids from small number of initiating cells and for their long-term culture.

To overcome the disadvantages of the current spheroid formation techniques, we developed a high throughput platform for screening human ovarian cancer therapies in a 384-hanging drop plate array. These plates feature arrays of holes instead of wells. A small droplet of liquid with cells can be placed in the access hole, creating a hanging drop, which stays in place due to surface tension. We start with a suspension of cells in cell culture medium in a 20 microliter droplet, which is dispensed into the access hole. Over time, the cells aggregate together, form cell-cell interactions, to create a multicellular structure known as spheroid. This platform is very user friendly, allows easy change of medium, and features easy addition of drugs and other factors directly onto the droplets. This platform has a wide appeal to a variety of other cancer cells, is useful for both tumor biology as well as drug screening studies.

Typically, only approximately 5000 ovarian CSC are obtained from flow cytometry sorting from a biopsy sample, which are barely enough to be used for only one experiment using standard conventional culture. We propose to optimize our culture system such that a maximum of 10 cells are necessary for sphere generation. Thus from a single patient sample, cells can be used more judiciously to conduct more experiments to understand tumorigenesis and drug-dose response. We will compare CSC spheroids formed with our platform to the standard mammosphere assay and other 3D assays. Since our platform is applicable to a wide variety of cells, we can systemically compare between CSC obtained using a variety of ovarian cancer stem cell markers as well as the side population cells. We will ultimately seed 1 CSC per spheroid, observe its clonal expansion over 4-6 weeks. These ovarian cancer stem cell (OvCSC) spheroids

will be maintained for up to 6 weeks in our platform and utilized for drug screening and xenograft studies.

The specific aims of this study are:

- Aim 1: Generate patient derived ovarian CSC spheroids with limiting numbers of initiating cells (as few as 1-10 cells).
- Aim 2: Quantify the drug-dose response of ovarian CSC treated with standard chemotherapy and novel drugs.
- Aim 3: Evaluate the functionality of spheroid grown tumor-initiating cells in mouse model.

Key Research Accomplishments

My lab's progress towards the specific aims of this grant are summarized below:

1) Generate OvCa spheroids with limiting numbers of initiating cells from OvCa cell lines.

As a first goal towards OvCSC spheroids, we have generated small cell number spheroids with OvCa cell lines and patient ascites, with the following cell types:

- Cell Lines:
 - SKOV3 GFP, SKOV3, SKTR (Taxane resistant SKOV3)
 - A2780 dsRED
 - OVCAR3 dsRED
 - CAOVS dsRED
- Patient derived ascites (from frozen ascites – not 2D cultured)

We have observed that SKOV3, OVCAR3, and A2780 spheroids can be formed with small cell numbers (Figure 2).

From the 3-D reconstructions of confocal z-stacks, it is clear that these small cell number spheroids show 3-D morphology (Figure 3).

We also observed differences in the expression of mesenchymal markers when ovarian cancer cells are grown within 3-D spheroids as compared to 2D cultures (Figure 4).

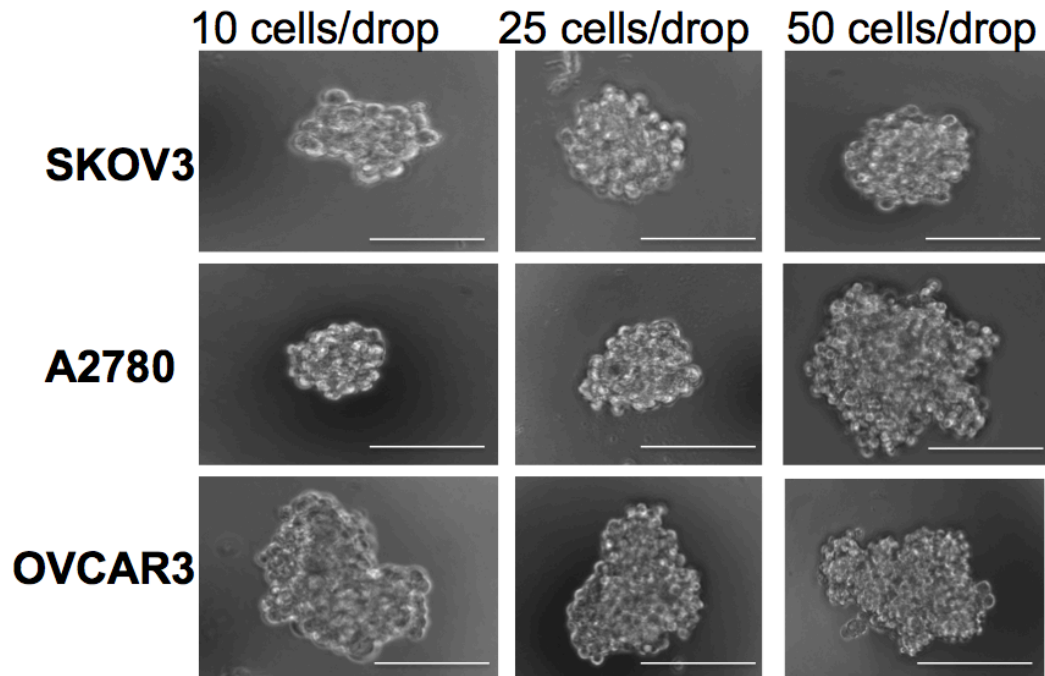


Figure 2: Ovarian cancer spheroids generated with small number of initial cells within the 384 hanging drop plate. Phase contrast image on day 4 after the cells are seeded onto the hanging drop plate. Scale bar =100 μ m.

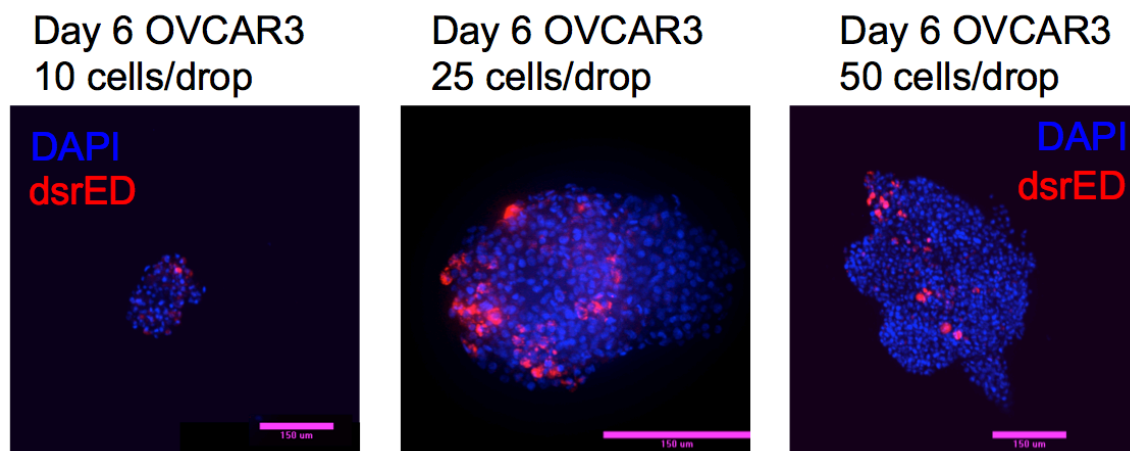


Figure 3: Small cell number spheroids demonstrate 3-D morphology. 3-D reconstructions of confocal z-stacks are shown for OVCAR3 dsRED spheroids generated with 10-50 cells.

Spheroids were harvested, encapsulated in agarose, imaged with a confocal microscope and the z-stacks were assembled together. Scale bar = 150 μ m.

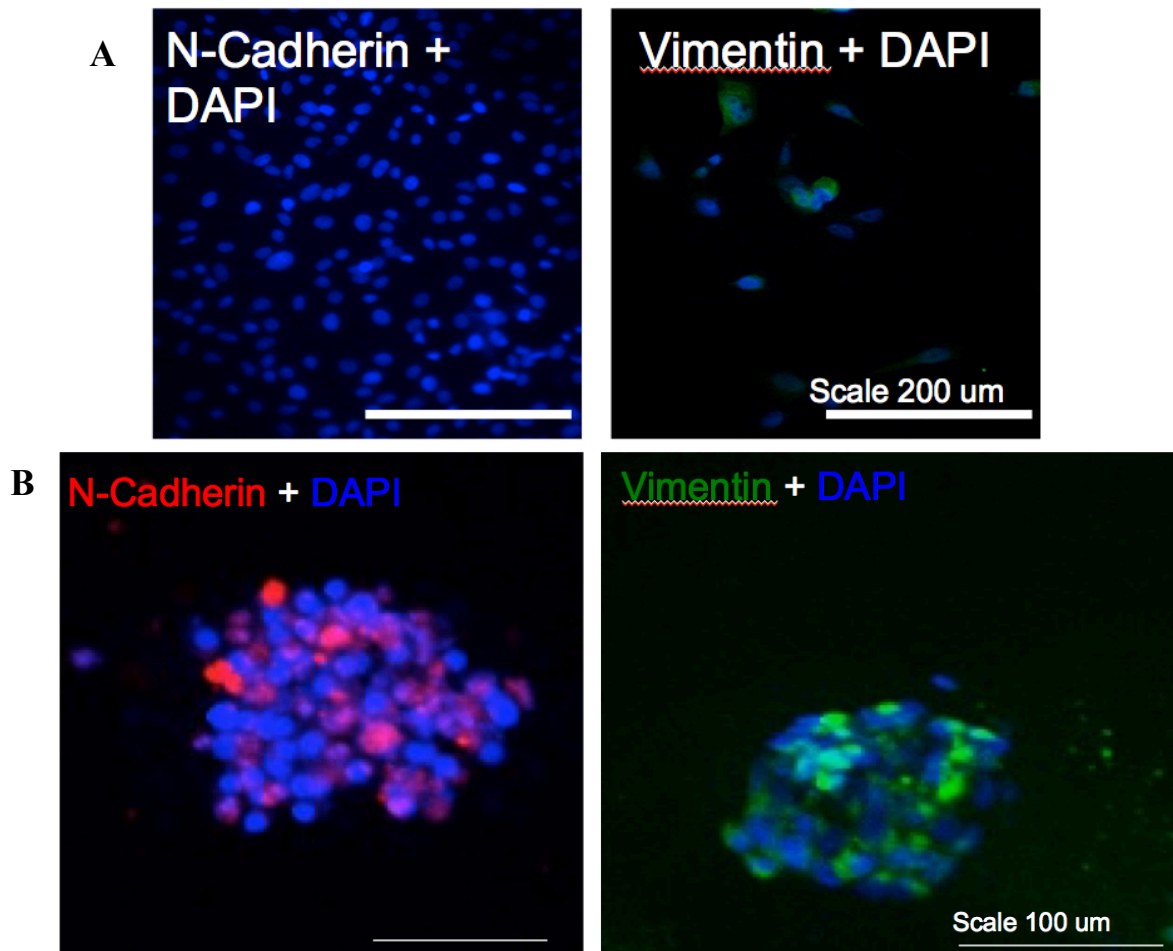


Figure 4: Epithelial cells in 3-D spheroids express mesenchymal markers N-Cadherin and Vimentin. Immunofluorescence images of SKOV3 cells in **A**) 2D and **B**) within spheroids at Day 7 obtained by confocal microscope, highlighting the appearance of N-Cadherin (red) and Vimentin (green) and nuclei (blue) within the spheroids. N-Cadherin was not observed in 2D culture. However, it can be prominently observed within spheroids. Vimentin expression also increased within the spheroids as compared to 2D. Scale bar = 200 μ m for 2D and 100 μ m for spheroids.

2) Generate OvCa spheroids with co-culture with MSCs or CA-MSCs.

MSC form the supportive microenvironment of OvCa *in vivo*. Studies have indicated that CA-MSC significantly enhance tumorigenesis by increasing CSC self-renewal. Moreover, MSC/CA-MSC increase cancer growth via an increase in CSCs, in part due to increased BMP2/4 and pSTAT3 signaling. Additionally, CA-MSC have a very different molecular profile than healthy donor control MSC. We add MSCs or CA-MSCs to the OvCa spheroids, to form a supportive microenvironment, reminiscent of the *in vivo* OvCa tumors.

Co-culture of OVCAR3 with MSCs helped form tighter spheroids when these cells were present in the ratios of 1:1, 1:3 and 1:6 OVCAR3:MSC (Figure 5).

In our experiments with CAOV3 cell line, we found that co-culture with MSCs helps make tighter spheroids and form CAOV3 spheroids (Figure 6).

CA-MSC provided survival and proliferation advantage to ovarian cancer cells. Comparison of Day 2, 5 and 9 compared to day 1 spheroids seeded with same initiating number and cell composition, illustrated that SKOV3:CA-MSC co-cultures have higher proliferation rates than SKOV3 or CA-MSC monocultures (Figure 7).

ALDH+ SKOV3 co-cultured with MSCs maintained spheroids for long term in serum free medium within the hanging drop plate (Figure 8). Since we aim to form spheroids with ovarian cancer stem cells grown in serum free medium, this is an important outcome for our ongoing work.

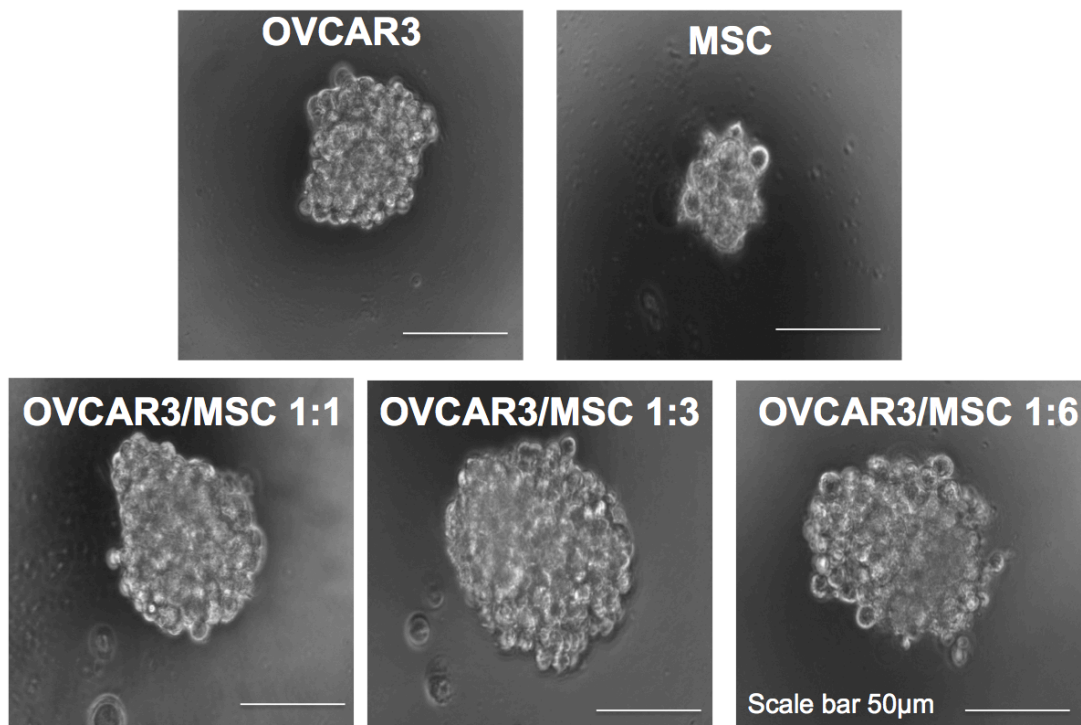


Figure 5: Co-culture of ovarian cancer cells with MSCs makes tighter spheroids. OVCAR3 cells were cultured with human adipose-derived mesenchymal stem cells in three different ratios (1:1, 1:3 and 1:6) with 50 cells/drop in 10% bovine serum containing medium. Phase micrographs shown on day 4. Tight sphere formation was observed with the addition of MSCs, and subsequent co-culture in hanging drop plates preserved spheroid morphology indicated in the phase micrographs. Scale bar = 50µm.

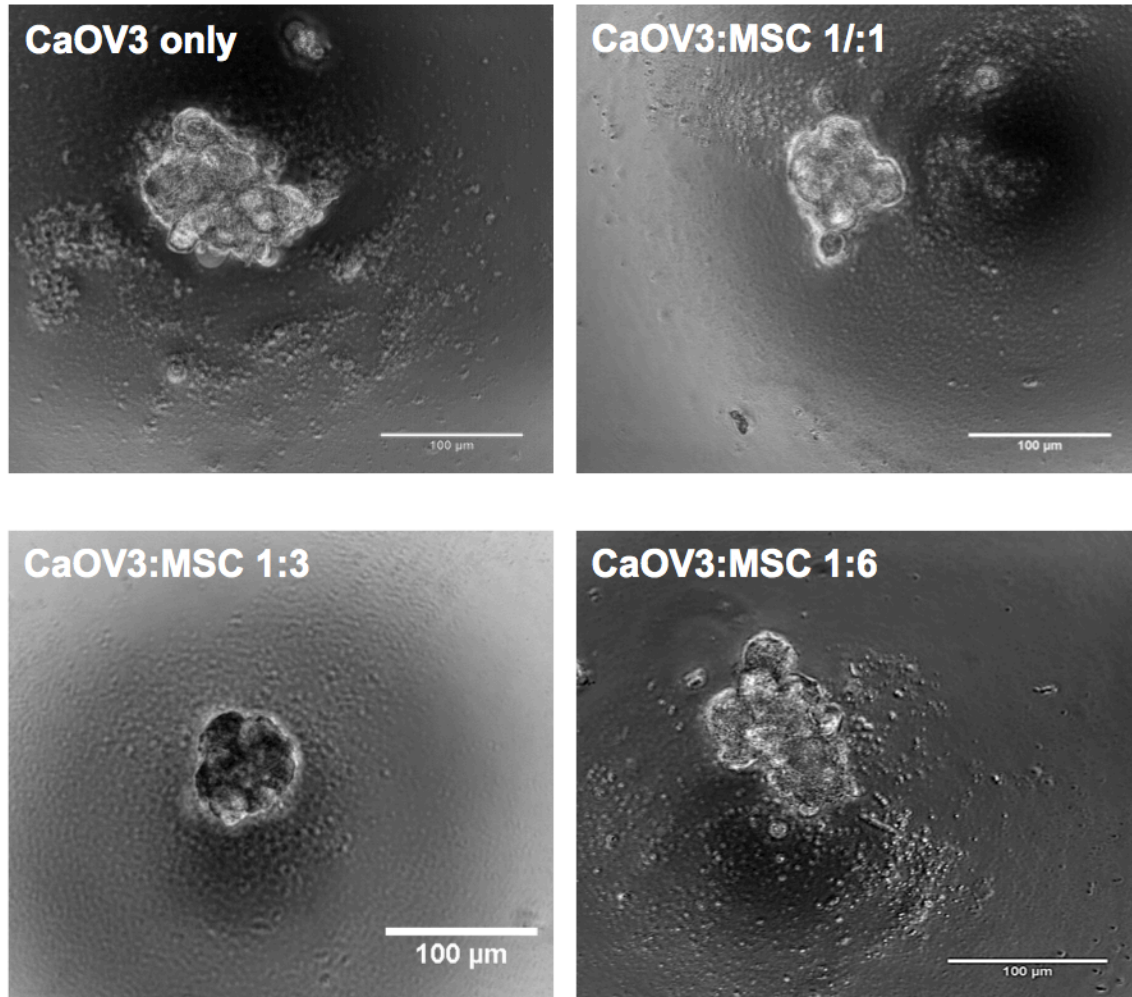


Figure 6: Co-cultured CAOV3 and MSC in serum free medium form tight spheroids. 50 cell spheroids were made to include two cell types: CaOV3 and MSCs. The ratios of the two cell types were varied as 1/0, 1/1, 1/3 and 1/6. Phase micrographs on day 2. Serum free medium is composed of 50/50 v/v DMEM/F12, 0.5X ITS, 1.5X Antibiotics/Antimycotics, 5ng/ml bFGF, 5ng/ml EGF, and 1X NEAA. Scale bar = 100μm.

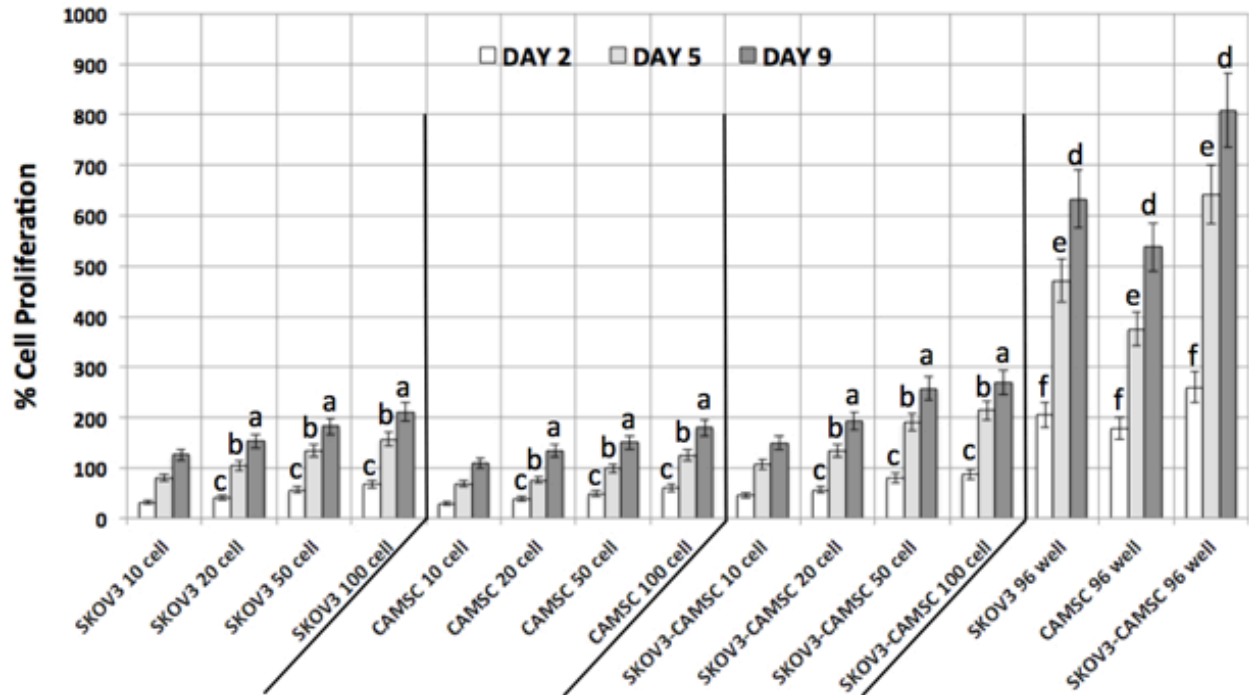


Figure 7: Co-cultured spheroids have higher proliferation rates. Percentage cell proliferation (increase in cell number) of SKOV3, CA-MSC and SKOV3-CAMSC (1:1) spheroids seeded with 10-100 cells, quantified by alamarblue on days 2, 5 and 9 after normalization with day 1 spheroids. Different letters between culture conditions (cell types, spheroid sizes or 2D) within a time point represent a significant difference between the spheroid sizes for each cell type (compared to 10 cell spheroid) or 2D (compared to same cell type at day1) (a, b, c, d, e, f = $p < 0.01$). CA-MSC provide survival and proliferation advantage to ovarian cancer cells.

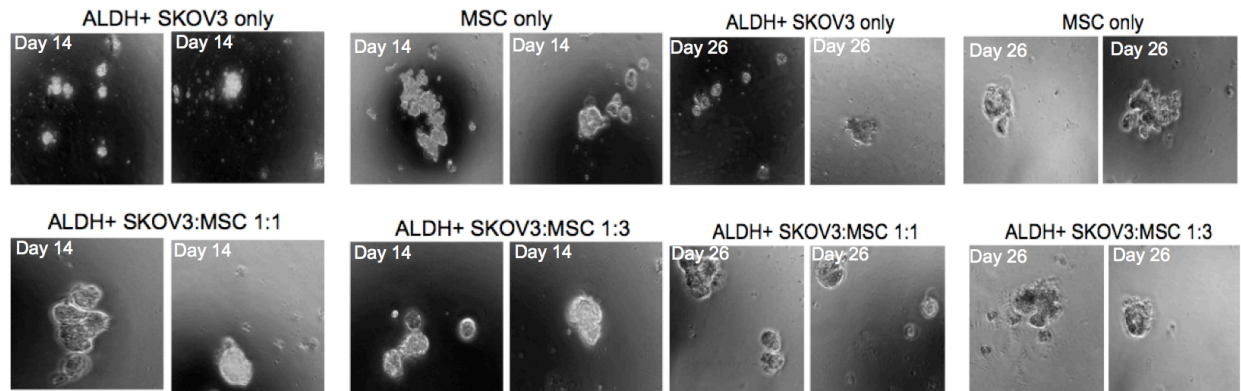


Figure 8: ALDH+ SKOV3 co-cultured with MSC maintain spheroids for long term. Phase micrographs from day 14 and day 26 for ALDH+ SKOV3 only, MSC only, 1:1 ALDH+ SKOV3: MSC, and 1:3 ALDH+ SKOV3: MSC maintained in serum free medium within the hanging drop plate. Serum free medium is composed of 50/50 v/v DMEM/F12, 0.5X ITS, 1.5X Antibiotics/Antimycotics, 5ng/ml bFGF, 5ng/ml EGF, and 1X NEAA.

3) Optimize medium composition for spheroids formed with patient ascites culture.

In order to form spheroids with patient ascites (from frozen ascites samples), we optimized the medium composition that would enable spheroid formation and growth. We tested the following media compositions:

- 10% RPMI: RPMI supplemented with 10% FBS, Pen Strep, Glutamine, and Gentamycin
- 5% IH: Ham's F-12 supplemented with 5% FBS, Insulin, Hydrocortisone, Pen Strep, Glutamine, and Gentamycin

- 5% HE: Ham's F-12 supplemented with 5% FBS, Hydrocortisone, EGF, Pen Strep, Glutamine, and Gentamycin
- 5% IHE: Ham's F-12 supplemented with 5% FBS, Insulin, Hydrocortisone, EGF, Pen Strep, Glutamine, and Gentamycin

With ascites alone, the 5% IH, HE and IHE media provided better formation of spheroids and growth of cells within these spheroids. However, the spheroids formed were not very tight, and cells existed as aggregates. When MSCs were added to the ascites, the co-culture helped make tighter spheroids.

We observed long-term maintenance of the CAOV3 spheroids in the serum free medium (Figure 9).

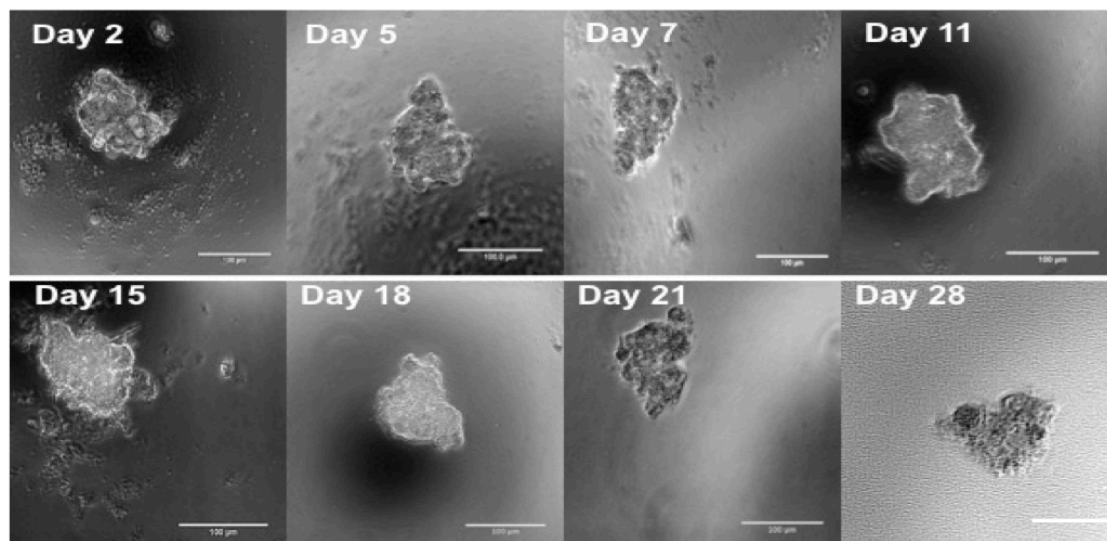


Figure 9: Long term culture of ovarian cancer spheroids in serum free growth medium on the 384 hanging drop plate. Tight spheroids of CAOV3 with clear demarcated boundaries were

formed, and cells were maintained up to 4 weeks as spheroids with no loss of 3D morphology. Phase contrast images on 100 cell spheroids at different time points. Serum free medium is composed of 50/50 v/v DMEM/F12, 0.5X ITS, 1.5X Antibiotics/Antimycotics, 5ng/ml bFGF, 5ng/ml EGF, and 1X NEAA. Scale bar =100 μ m.

4) Quantify the drug-dose response of ovarian CSC treated with standard chemotherapy and novel drugs.

We screened the drug-dose response of OvCa spheroids with doxil, cisplatin and gemcitabine (Figure 10, 11, 12, 13). Viability of cells in parental and taxane resistant (SKTR) SKOV3 spheroids decreases with decreasing drug dose. Cell viability (%) on day 7 quantified using the alamarblue assay, normalized to non-treated SKOV3 spheroids on Day7. Chemosensitivity of spheroids was directly compared with chemosensitivity of cells grown in 2D culture. We treated the spheroids with a wide range of concentrations of standard ovarian cancer therapy drugs (doxil, gemcitabine, cisplatin) from days3-7 after seeding cells into the HDs and quantified the cell viability (on day7) using alamarblue assay and normalized to non drug-treated spheroids on day7. Size and viability of cells in the spheroids declined with increasing doxil concentration, however, taxane resistant SKOV3 exhibited resistance to doxil treatment, and statistically significant higher viability compared to parental SKOV3. Importantly, compared to 2D culture, spheroids from as few as 10 cells demonstrated higher resistance to doxil treatment at all concentrations for both parental and taxane resistant SKOV3. Moreover, consistent with a better ability to replicate *in vivo* physiology, co-culture with CAMSCs provided an even greater

survival advantage to the SKOV3 spheroids with respect to drug treatment (compared to SKOV3 alone or CAMSCs alone).

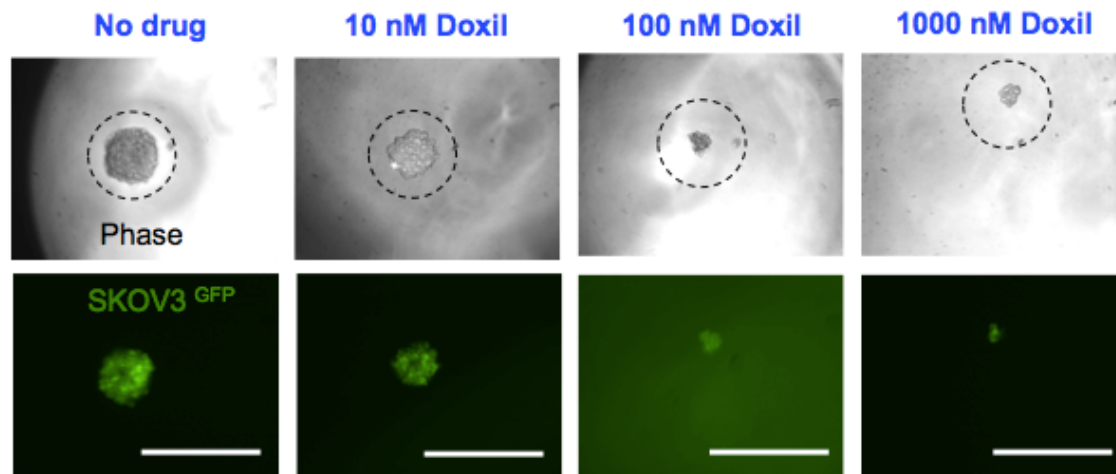


Figure 10: Drug screening on the small cell spheroids. SKOV3 GFP-CAMSC spheroids were treated with 10-1000 nM of doxil from day 3-7 of culture. Representative phase and GFP images of the 10 cell spheroids on day 7 after treatment with increasing dose of doxil. Scale bar = 200 μ m.

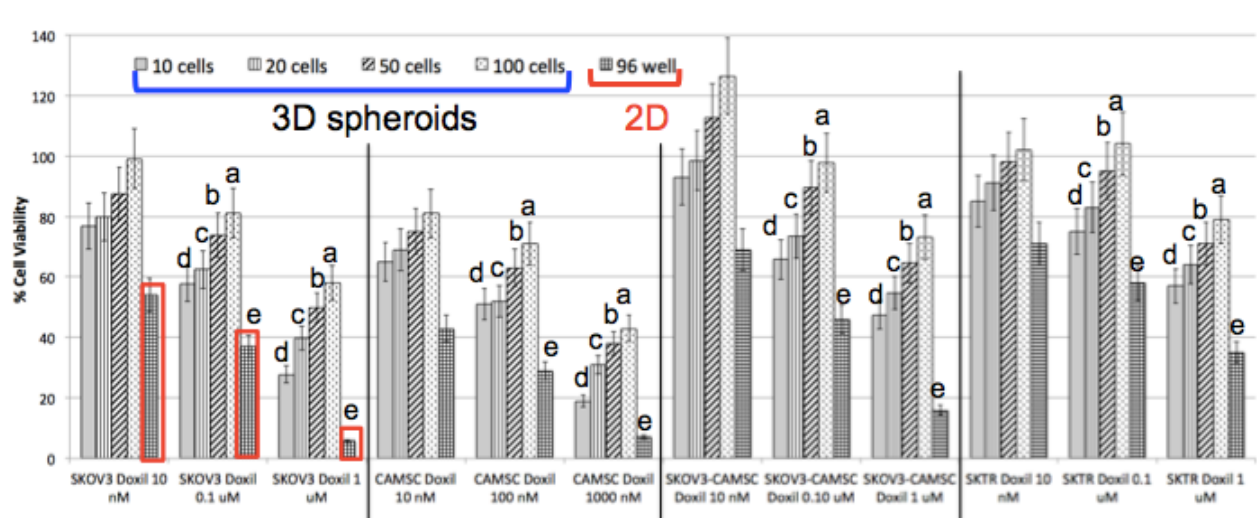


Figure 11: Quantification of drug-dose response of ovarian cancer spheroids treated with doxil. Viability of cells in parental and taxane resistant (SKTR) SKOV3 spheroids decreases

with decreasing drug dose. Cell viability (%) on day 7 quantified using the alamarblue assay, normalized to non-treated SKOV3 spheroids on Day7. Different letters between culture conditions (cell type, spheroid sizes or 2D) for a doxil dose represent a significant difference between the spheroid sizes for each cell type or 2D (compared to 10 nm doxil) (a, b, c, d, e = $p < 0.01$, $n > 3$). 96 well refers to the conventional 2D control, with 5000 cells in each 96 well.

Chemosensitivity of spheroids was directly compared with chemosensitivity of cells grown in 2D culture. We treated the spheroids with a wide range of concentrations of standard ovarian cancer therapy drugs (doxil, gemcitabine, cisplatin) from days3-7 after seeding cells into the HDs and quantified the cell viability (on day7) using alamarblue assay and normalized to non drug-treated spheroids on day7. Size and viability of cells in the spheroids declined with increasing doxil concentration (Figure 10, 11), however, taxane resistant SKOV3 exhibited resistance to doxil treatment, and statistically significant higher viability compared to parental SKOV3. Importantly, compared to 2D culture, spheroids from as few as 10 cells demonstrated higher resistance to doxil treatment at all concentrations for both parental and taxane resistant SKOV3. Moreover, consistent with a better ability to replicate *in vivo* physiology, co-culture with CAMSCs provided an even greater survival advantage to the SKOV3 spheroids with respect to drug treatment (compared to SKOV3 alone or CAMSCs alone). We obtained similar results with cisplatin and gemcitabine (Figure 12 and 13).

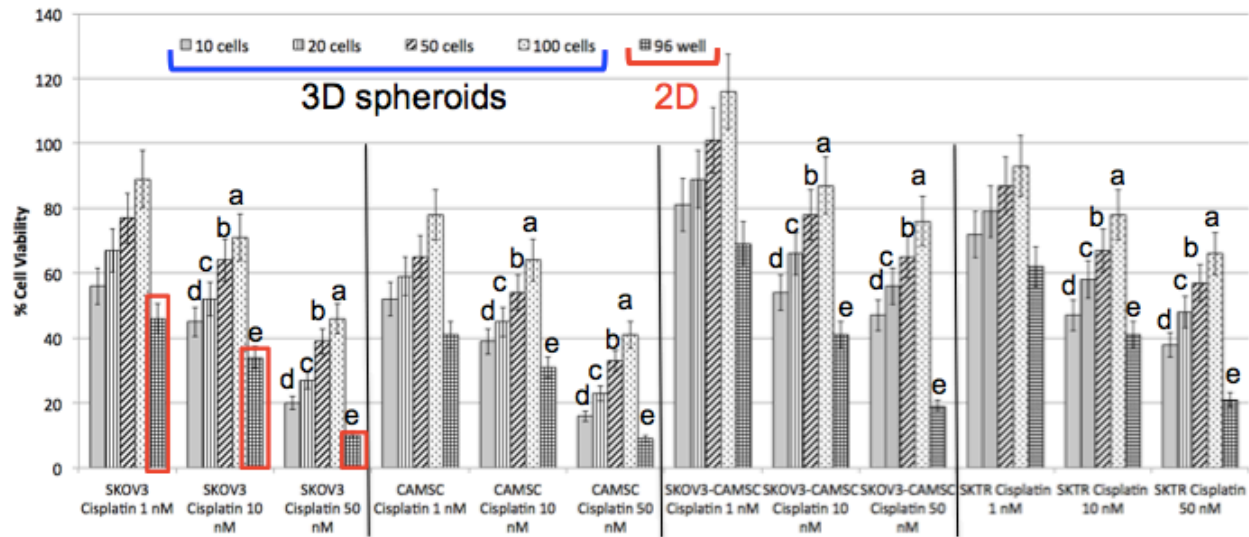


Figure 12: Quantification of drug-dose response of ovarian cancer spheroids treated with cisplatin. Viability of cells in parental and taxane resistant (SKTR) SKOV3 spheroids decreases with decreasing drug dose. Cell viability (%) on day 7 quantified using the alamarblue assay, normalized to non-treated SKOV3 spheroids on Day7. Different letters between culture conditions (cell type, spheroid sizes or 2D) for a cisplatin dose represent a significant difference between the spheroid sizes for each cell type or 2D (compared to 10 nm doxil) (a, b, c, d, e = $p < 0.01$, $n > 3$). 96 well refers to the conventional 2D control, with 5000 cells in each 96 well.

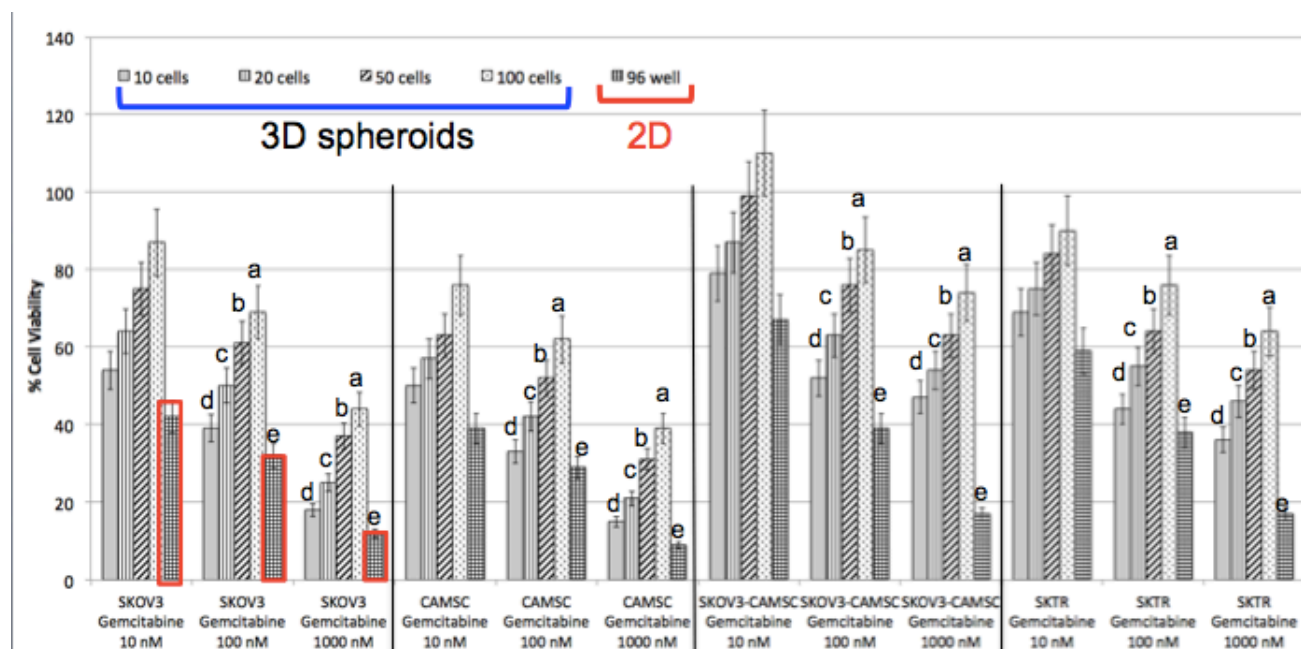


Figure 13: Quantification of drug-dose response of ovarian cancer spheroids treated with gemcitabine. Viability of cells in parental and taxane resistant (SKTR) SKOV3 spheroids decreases with decreasing drug dose. Cell viability (%) on day 7 quantified using the alamarblue assay, normalized to non-treated SKOV3 spheroids on Day7. Different letters between culture conditions (cell type, spheroid sizes or 2D) for a gemcitabine dose represent a significant difference between the spheroid sizes for each cell type or 2D (compared to 10 nm doxil) (a, b, c, d, e = $p < 0.01$, $n > 3$). 96 well refers to the conventional 2D control, with 5000 cells in each 96 well.

We were able to establish that the small cell number spheroids are efficient model for chemoresistance compared to 2D. Moreover, we observed that CA-MSC provide chemoresistance to ovarian cancer spheroids. Therefore, we have created a platform that enables

drug resistance and sensitivity to be accurately assessed for ovarian cancer cells within 3D spheroids.

5) Generating small cell number patient derived OvCSC spheroids.

There are many challenges associated with translating our work with OvCa cell lines to generate small cell number patient derived OvCSC spheroids. Theoretically, with CSCs, single cell can form spheroids by monoclonal growth, however the cell-cell contacts are needed for aggregation and spheroid formation. It is often difficult to titrate and manipulate cell suspensions into single cell levels. Moreover, CSCs may have undergone stress through isolation and sorting, and may not fare well in the 3-D spheroid cultures. For anchorage-dependent cells such as ovarian epithelial cells, lack of cell-cell interaction slows down proliferation, and may even lead to cell death.

In an effort to generate small cell number patient derived OvCSC spheroids, we are investigating the following approaches:

- Addition of non-cell instructive hydrogels, such as methylcellulose and agarose, to cell suspensions, in order to enhance spheroid assembly by providing cell-matrix support.
- Co-culture small numbers of CSCs with other supporting cell types.
- Omniphobic surfaces (Tuteja Lab) for long term spheroid stability and robustness.

We are also working on generating spheroids with 2, 5 and 10 cells from ascites co-cultured with MSCs or CA-MSCs.

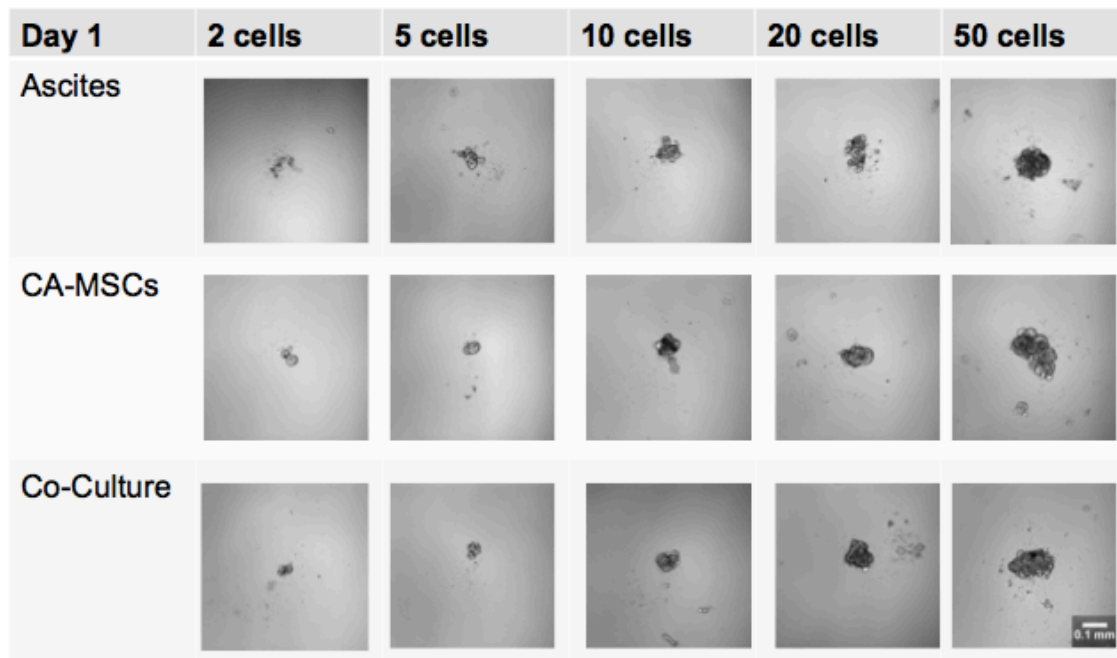


Figure 14: Generating small cell number patient spheroids. Spheroids initiated with 2, 5, 10, 20 and 50 cells from patient ascites only, CA-MSCs only or 1:1 ascites: CA-MSCs. Representative phase images of the spheroids on day 1. Scale bar = 100 μ m.

We have established small cell number spheroids with patient ascites (Figure 14 and 15).

We have also demonstrated that more than 90% of the patient derived cells within the small cell number spheroids are live (Figure 16).

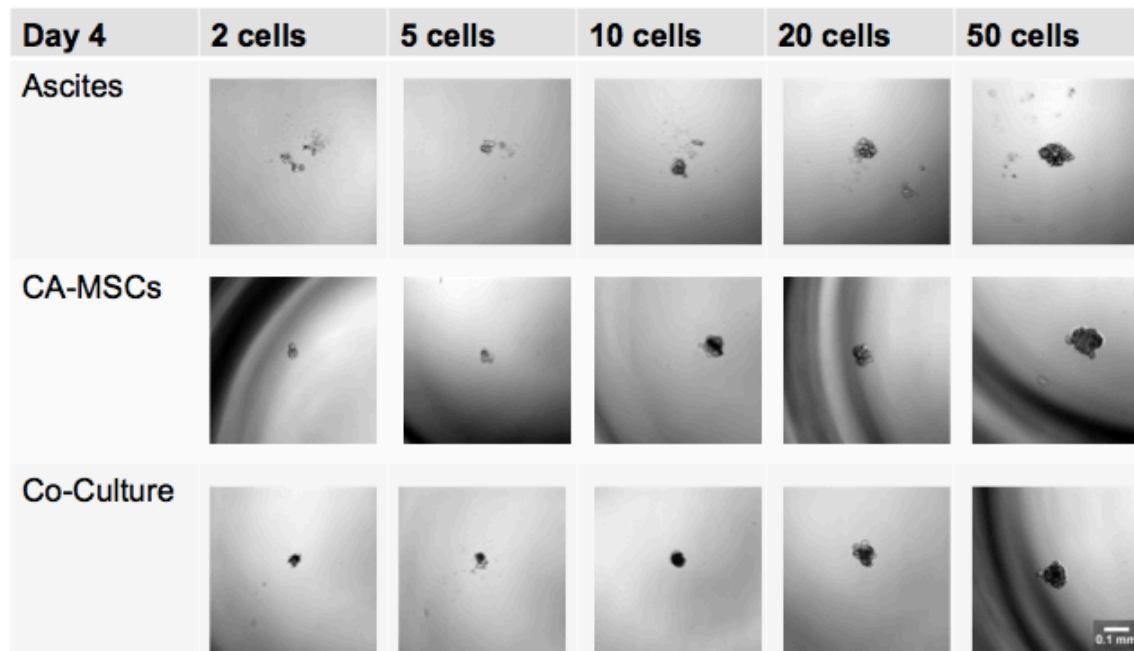


Figure 15: Maintaining small cell number patient spheroids. Spheroids initiated with 2, 5, 10, 20 and 50 cells from patient ascites only, CA-MSCs only or 1:1 ascites: CA-MSCs. Representative phase images of the spheroids on day 4. Scale bar = 100 μ m.

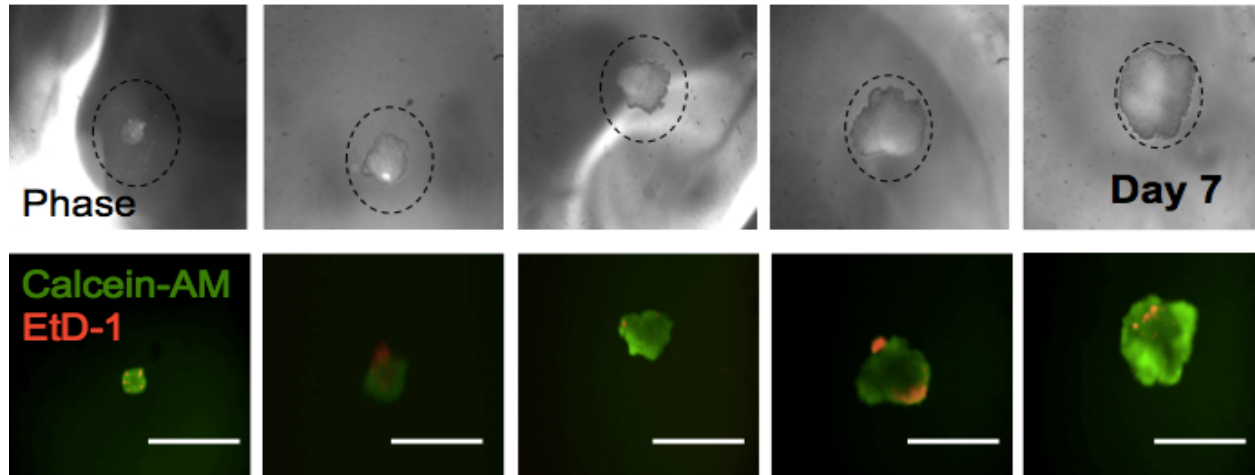


Figure 16: Spheroids from patient derived cells contain viable cells. Phase and fluorescent images of live-dead (Calcein-AM and Ethidium homodimer) stained images of spheroids seeded with 10-50 cells on day 7 (left to right: 10, 20, 30, 40, 50 cell). Scale bar = 200 μ m.

Our plans for the future experiments include:

- Serum free medium for all OvCa spheroids (including cell lines, patient ascites, and FACS sorted CSCs).
- Check the functionality of OvCSCs after being cultured in spheroids.
- Serial passaging of spheroids.
- Check if aggregates/spheroids contain stem cell-marker negative (for example, CD133-negative cells), to indicate some degree of differentiation within the culture.
- Since cancer stem cells are functionally defined and markers are only good for isolation and to some extent good for demonstration of differentiation capacity, xenograft models will be utilized to demonstrate that OvCSC growing in spheroids isolated have the tumor initiating property.

Conclusion

We have made several important research findings originated by the Ovarian Cancer Academy ECI Award. These include:

- A)** Establishment of high throughput novel hanging drop array plate, for generation of human ovarian cancer (OvCa) spheroids *in vitro* that resemble tumors *in vivo*.
- B)** Generation of three-dimensional spheroids with small cell numbers from several OvCa cell lines, as well as, from individual patient derived ascites.
- C)** Co-culture with mesenchymal stem cells (MSCs) enables formation of tighter spheroids, promotes higher proliferation rates and provides a survival advantage to OvCa cells.
- D)** Optimized several nutrient compositions that could eliminate the need for animal-derived serum while promoting the formation and growth of spheroids from patient ascites.
- E)** Screened the drug-dose response of OvCa spheroids with doxil, cisplatin, gemcitabine, and novel drug targets.
- F)** Viability of OVCAR3, A2780, parental and taxane resistant SKOV3 spheroids decreases with increasing drug dose in spheroids.
- G)** Compared to 2D culture, spheroids from as few as 10 cells demonstrated higher resistance to drug treatment at all concentrations.
- H)** Co-culture with MSCs provides an even greater survival advantage to the OvCa spheroids with respect to drug treatment.

Publications, Abstracts and Presentations

- 1) "In vitro 3-D Model of Ovarian Cancer for High Throughput Drug Screening and Personalized Medicine", Erin Flannery, Kathleen M Woods Ignatoski, Geeta Mehta, National Conference on Undergraduate Research (NCUR) 2014 at University of Kentucky, Lexington, KY, April 3-5, 2014. (Poster)
- 2) "In vitro 3-D Model of Ovarian Cancer for High Throughput Drug Screening and Personalized Medicine", Erin Flannery, Kathleen M Woods Ignatoski, Geeta Mehta, Michigan Research Community Program (MRC) 2014 Annual Spring Research Symposium at the University of Michigan, Ann Arbor, MI, April 9, 2014. (Poster)
- 3) "3-D Culture in Hydrogels to Study Microenvironmental Factors in HER2 Expression in Breast Cancer", Alyssa Cramer, Kathleen M Woods Ignatoski, Geeta Mehta, Undergraduate Research Opportunity Program (UROP) 2014 Annual Spring Research Symposium at the University of Michigan, Ann Arbor, MI, April 23, 2014. (Poster)
- 4) "Study of Effects of Compressive Forces on the Differentiation of Hematopoietic Stem and Progenitor Cells", Yusuf Ghani, Kathleen M. Woods Ignatoski, Geeta Mehta, Undergraduate Research Opportunity Program (UROP) 2014 Annual Spring Research Symposium at the University of Michigan, Ann Arbor, MI, April 23, 2014.

(Poster)

- 5) “Formation of Stable Small Cell Number Ovarian Cancer Spheroids for Individualized Therapy”, Geeta Mehta, Shreya Raghavan, Brittany Gnewkowski, YanChen Liu, Lourdes Cabrera, Shu Takayama, Ronald J Buckanovich, 10th Biennial Ovarian Cancer Research Symposium, September 8-9, 2013 Seattle, Washington. (Poster)
- 6) “Formation of Stable Small Cell Number Ovarian Cancer Spheroids for Individualized Therapy”, Shreya Raghavan, Maria Ward, Katelyn Rowley, Ronald J. Buckanovich, Geeta Mehta, 2014 Annual University of Michigan Comprehensive Cancer Center Research Symposium, October 24, 2014, Ann Arbor, MI. (Poster)

Inventions, Patents and Licenses

None

Reportable Outcomes

I am planning to write multiple manuscripts based on the research that my lab has conducted thus far. Our first manuscript based on this work is in review in *Gynecologic Oncology*.

Our future manuscripts will feature the following outcomes:

1) OvCa spheroids with limiting numbers of initiating cells from OvCa cell lines

We have optimized the hanging drop array culture system to generate spheroids with small cell numbers from several OvCa cell lines as well as two individual patient derived ascites. Confocal microscopy indicates that small cell number spheroids demonstrate three-dimensional morphology.

2) OvCa spheroids with co-culture with Mesenchymal stem cells (MSCs)

Our results demonstrated that tumor spheroids from the serous cell line CaOV3 form tighter spheroids when co-cultured with MSCs compared to monoculture. Furthermore, co-culture promoted higher proliferation rates and provided a survival advantage to OvCa cells.

3) Medium composition for spheroids formed with patient ascites culture

We tested several nutrient compositions that could eliminate the need for animal-derived serum while promoting the formation and growth of spheroids from patient ascites. Media supplemented with insulin, hydrocortisone and EGF maintained tumor spheroids and supported growth well. Co-culture with MSCs and ascites driven cells resulted in tight spheroid formation.

4) Drug-dose response of ovarian CSCs

We screened the drug-dose response of OvCa spheroids with doxil, cisplatin and gemcitabine. Viability of cells in parental and taxane resistant (SKTR) SKOV3 spheroids decreases with decreasing drug dose. Compared to 2D culture, spheroids from as few as 10 cells demonstrated higher resistance to doxil treatment at all concentrations for both parental and taxane resistant SKOV3. Moreover, consistent with a better ability to replicate *in vivo* physiology, co-culture with CA-MSCs provided an even greater survival advantage to the SKOV3 spheroids with respect to drug treatment (compared to SKOV3 alone or CAMSCs alone).

Other Achievements

Scientific Meetings attended:

- 10th Biennial Ovarian Cancer Research Symposium, September 8-9, 2013 Seattle, Washington.
- Undergraduate Research Opportunity Program (UROP) 2014 Annual Spring Research Symposium at the University of Michigan, Ann Arbor, MI, April 23, 2014.
- Michigan Research Community Program (MRC) 2014 Annual Spring Research Symposium at the University of Michigan, Ann Arbor, MI, April 9, 2014.
- Annual University of Michigan Comprehensive Cancer Center Research Symposium, October 24, 2014, Ann Arbor, MI.

Awards, memberships:

1. Member: University of Michigan Comprehensive Cancer Center, University of Michigan
2. Member: Center for Organogenesis, University of Michigan
3. Appointment: Division of Macromolecular Science and Engineering, University of Michigan
4. Service: Mentor to the students in the Undergraduate Research Opportunities Program (UROP), Michigan Research Community Program (MRC), and Marian Sarah Parker Scholars Program, at the University of Michigan. All of these programs are catered towards undergraduate involvement in academic research.

5. Service for my home department: I serve on the following committees in the department of Materials Science and Engineering (MSE): MSE Seminar Committee, MSE Graduate Committee, MSE Curriculum Committee
6. Service for College of Engineering: I serve on the Faculty Committee on Discipline

Manuscripts (including journal, and status, i.e., submitted/under review/published):

1. A. R. Dixon, S. Rajan, C.-H. Kuo, T. Bersano, R. Wold, N. Futai, S. Takayama, **G. Mehta#**, “Microfluidic Device Capable of Medium Recirculation for Non-Adherent Cell Culture”, *Biomicrofluidics*. (2014). 8, 016503, 1-14. DOI: 10.1063/1.4865855. (# corresponding author) (Impact Factor: 3.4)
2. **G. Mehta#**, Y. Shiozawa, R. S. Taichman, “Hematopoietic Stem Cells and their niches”, in *Biomaterials and Regenerative Medicine*, P. Ma (Editor), Cambridge University Press, (2014) (# corresponding author) (Book Chapter)
3. **G. Mehta#**, M. Davis, H. Rios, Y. Shiozawa, Y. Mishina, K. H. Kozloff, R. S. Taichman, “Hematopoietic stem cell (HSC) activity is modulated by mechanical stimulation”, (2014), *Stem Cells and Development*, In Review (# corresponding author) (Impact Factor: 4.2)

4. S. Raghavan, M. Ward, K. Rowley, R. J. Buckanovich, **G. Mehta**[#], “Formation of Robust, Stable, and High Throughput Small Cell Number Ovarian Cancer Spheroids for Individualized Therapy”, , *In Preparation*. ([#] corresponding author)

Promotions:

Promoted from Assistant Research Scientist in the Department of Biomedical Engineering to Tenure Track assistant Professor in Departments of Materials Sciences and Engineering and Biomedical Engineering at the University of Michigan, Ann Arbor (09/01/2013).

Recently established collaborations and resource sharing:

- My lab has a share in the confocal microscope (shared resource) with Dr. Allen Liu in Mechanical Engineering, University of Michigan, Ann Arbor, MI.
- Established collaboration with Dr. Anish Tuteja (Assistant Professor in MSE, Chemical Engineering and Macromolecular Science and Engineering, University of Michigan), to use omniphobic surfaces pioneered in his lab for generating ovarian cancer spheroids.
- Established collaboration with Dr. Max Shtein (Associate Professor in MSE, Physics, Chemical Engineering and Macromolecular Science and Engineering, University of Michigan), to use chemical vapor jet deposition technique pioneered in his lab for creating drug films with low solubility in aqueous solutions, which can target cancer cells.

Participation in manuscript reviews or grant reviews:

- Reviewer for the following Journals: Stem Cells, Scientific Reports, Nanomedicine, Biomaterials, Tissue Engineering: Part A, Annals of Biomedical Engineering, Analytical Chemistry, Lab on a Chip, Journal of Biomedical Optics, Journal of Biotechnology,

Biotechnology Progress, Journal of Biomedical Materials Research: Part A, Journal of Biomechanical Engineering, Acta Biomaterialica

- Reviewer for: University of Michigan Internal Grants administered by the Office of Research

ADVISING AND MENTORING – selected – full list available on request

Graduate Student Mentorship

Sarah Snyder, Materials Science and Engineering

Radha Arghal, Biomedical Engineering

Katarina Klusman, Biomedical Engineering

Nicholas Cobane, Biomedical Engineering

Caymen Novak, Biomedical Engineering

Nathan Tinetti, Biomedical Engineering

Pratik Holla, Biomedical Engineering

Undergraduate Student Mentorship

Eric Horst, Materials Science and Engineering

Katelyn Rowley, Biomedical Engineering

Dylan Zwiers, Materials Science and Engineering

Erin Flannery, Materials Science and Engineering

Alyssa Cramer, Biomedical Engineering

Yusuf Ghani, Biomedical Engineering

Rachel Wold, Biomedical Engineering

Jonathon Muncie, Biomedical Engineering

Martin Sisolak, Materials Science & Engineering

Changqi Dai, Biomedical Engineering

Anjali Sood, Microbiology

Technician Mentorship

Maria Ward

Brittany Gnewkowski

Program Mentor

Women in Science and Engineering (WISE), University of Michigan

Undergraduate Research Opportunity Program (UROP), University of Michigan

Michigan Research Community (MRC), University of Michigan

Marian Sarah Parker Scholars Program, University of Michigan

Summer High School Students Mentorship

Raghu Arghal (Novi High School, Novi, MI) - Summer 2014

Randy Strassburg (Skyline High School, Ann Arbor, MI) - Summer 2014

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Appendices

None